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(21) International Application Number: PCT/EP95/04480 (22) International Filing Date: 14 November 1995 (14.11.95) (30) Priority Data: 08/339,985 14 November 1994 (14.11.94) US (71) Applicant (for all designated States except AT/DE): SANDOZ LTD. [CH/CH]; Lichtstrasse 35, CH-4002 Basle (CH). (71) Applicant (for DE only): SANDOZ-PATENT-GMBH [DE/DE]; Humboldtstrasse 3, D-79539 Lörrach (DE). (71) Applicants (for AT only): SANDOZ-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT MBH [AT/AT]; Brunner Strasse 59, A-1230 Vienna (AT). SYSTEMIX, INC. [US/US]; 3155 Porter Drive, Palo Alto, CA 94304 (US). (72) Inventor: WICKREMA, Amittha; 842 Greennew Drive, Mountain View, CA 94040 (US). (74) Common Representative: SANDOZ LTD.; Patents & Trade-marks Div., Lichtstrasse 35, CH-4002 Basle (CH).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: ASSAY FOR THE MEASUREMENT OF MULTILINEAGE STEM CELLS AND METHODS OF USE THEREOF		
(57) Abstract <p>The present invention encompasses methods of detecting and quantitating hematopoietic stem cells in a population of hematopoietic cells. A population of hematopoietic cells is first obtained and treated to enrich for stem cells. Single cells in the enriched population are deposited onto a suitable growth substrate such as a well in a 96 well microtiter plate. The single cell deposits are cultured under conditions suitable to maintain viability and induce differentiation. The resultant populations are then assayed for the expression of lineage-specific messenger RNA transcripts specific to at least two different lineages. The presence of transcripts specific to two or more different lineages is scored as positive for the initial presence of a stem cell.</p>		

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Assay for the Measurement of Multilineage
Stem Cells and Methods of Use Thereof

Field of the Invention

This invention provides methods for determining the presence of hematopoietic stem cells in a population of hematopoietic cells.

Background of the Invention

Mammalian hematopoietic cells provide a diverse range of physiologic activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes, as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

Despite the diversity of the nature, morphology, characteristics and function of hematopoietic cells, it is presently believed that these cells are derived from a single cell population, termed "stem cells." Unlike more "mature" blood cells, stem cells are capable of self-regeneration but may also divide into progenitor cells that are no longer pluripotent and capable of self-regeneration. These

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progenitor cells divide repeatedly to form more mature cells which eventually become terminally differentiated to form the various mature hematopoietic cells. Thus the large number of mature hematopoietic cells is derived from a small reservoir of stem cells by a process of proliferation and differentiation. As used herein, "stem cells" refers to hematopoietic stem cells and not stem cells of other cell types.

Stem cells mature into progenitor cells and then become lineage committed, that is, are incapable of maturing into all of the three lineages. The use of the words progenitor or progenitor cells indicates cell populations which are no longer stem cells but which have not yet become terminally differentiated. The use of the word lymphoid, myeloid or erythroid in conjunction with progenitor indicates the potential cell populations into which the progenitor is capable of maturing.

A highly purified or enriched population of stem cells is necessary for a variety of *in vitro* experiments and *in vivo* indications. For instance, a purified population of stem cells will allow for identification of growth factors associated with their self-regeneration. In addition, there may be as yet undiscovered growth factors associated with: (1) the early steps of dedication of the stem cell to a particular lineage; (2) the prevention of such dedication; and (3) the negative control of stem cell proliferation.

Stem cells find use in: (1) regenerating the hematopoietic system of a host deficient in any class of hematopoietic cells; (2) a host that is diseased and can be treated by removal of bone marrow, isolation of stem cells and treatment with drugs or irradiation prior to re-engraftment of stem cells; (3) producing various hematopoietic cells; (4) detecting and evaluating growth factors relevant to stem cell self-regeneration; and (5) the

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development of hematopoietic cell lineages and assaying for factors associated with hematopoietic development.

Stem cells are also important targets for gene therapy, where expression of the inserted genes promotes the health of the individual into whom the stem cells are transplanted. In addition, the ability to isolate stem cells may serve in the treatment of lymphomas and leukemias, as well as other neoplastic conditions where the stem cells are purified from tumor cells in the bone marrow or peripheral blood, and reinfused into a patient after myelosuppressive or myeloablative chemotherapy. Thus, there have been world-wide efforts toward isolating stem cells in substantially pure or pure form.

Stem cells and progenitor cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface protein or carbohydrate "markers." Such markers may be either specific to a particular lineage or be present on more than one cell type. The markers also change with stages of differentiation. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem and progenitor cells. One marker which was previously indicated as present solely on stem cells, CD34, raised against KG1a cells, is also found on a significant number of lineage committed progenitors. U.S. Patent No. 4,714,680 describes a composition comprising human CD34⁺ stem and progenitor cells.

The CD34 marker is found on numerous lineage committed hematopoietic cells. In particular, 80-90% of the CD34⁺ population is marked by other lineage specific and non-specific markers. Therefore, in view of the small proportion of the total number of cells in the bone marrow or peripheral blood which are stem cells, the uncertainty of the markers associated with the stem cell as distinct from more differentiated cells, and the general difficulty in assaying

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for human stem cells biologically, the identification and purification of stem cells has been elusive.

Characterizations and isolation of human stem cells are reported in: Baum et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2804-2808; and Tsukamoto et al. U.S. Patent No. 5,061,620.

Hematopoietic cells can be characterized by the level of rhodamine 123 (rho123) staining, which is determined not by the initial dye accumulation but by an efflux process sensitive to P-glycoprotein (P-gp) inhibitors. Retention of several P-gp-transported fluorescent dyes, including rho123, in human bone marrow cells was inversely correlated with the expression of P-gp. Bone marrow cells expressing physical and antigenic characteristics of pluripotent stem cells showed high levels of P-gp expression and fluorescent dye efflux, i.e. are rhodamine low. Fractions of human bone marrow cells isolated on the basis of either increased rho123 efflux or P-gp expression contained practically all the primitive progenitor cells of human bone marrow, including long-term culture-initiating cells (LTCIC). Chaudhary and Roninson (1991) *Cell* 66:85-94.

Recently, the mouse stem cell has been obtained in at least highly concentrated, if not a purified form, where fewer than about 30 cells obtained from bone marrow were able to reconstitute all of the lineages of the hematopoietic system of a lethally irradiated mouse. Each assayed cell is multipotent for all hematopoietic lineages, while self-renewal is variable amongst these cells. Spangrude et al. (1988) *Science* 241:58-62; Smith et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2788-2792; Uchida (1992) *Ph.D. Thesis* Stanford U.; and see also, EPA 89 304651.6 and the references cited therein which describe the isolation of mouse stem cells.

In contrast to the mouse system, studies of human hematopoietic stem cells have been hampered due to the limitations of stem cell assays. Two in vivo assays which

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have proved very useful in the study of human stem cells are the SCID-hu bone and SCID-hu thymus assays.

The SCID-hu bone model allows the detection of myeloid and B-lymphoid cell potential in a putative stem cell population. Kyoizumi et al. (1992) *Blood* 79:1704. To analyze this, one may isolate human fetal bone and transfer a longitudinally sliced portion of this bone under the skin of a scid/scid animal: the bone cavity is diminished of endogenous cells by whole body irradiation of the mouse host prior to infection of the test donor population. The HLA of the population which is injected is mismatched with the HLA of the host bone cells. Stem cells from human hematopoietic sources have been found to engraft and sustain B lymphopoiesis and myelopoiesis in the SCID-hu bone model.

The SCID-hu thymus model allows the detection of T-cell potential in a test population. To demonstrate differentiation T cells, fetal thymus is isolated and cultured for from 4-7 days at about 25°C, so as to deplete substantially the lymphoid population. The cells to be tested for T cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue may then be transplanted into a scid/scid mouse as described in U.S. Patent No. 5,147,784, particularly transplanting under the kidney capsule. After 6-10 weeks, assays of the thymus fragments injected with the test cells can be performed and assessed for donor-derived T cells.

Two *in vitro* assays that are believed to correlate with stem cell activity *in vivo* each require 5-7 weeks to perform. The long-term culture initiating cell (LTC-IC) limiting dilution assay requires an initial 5 week stromal cell coculture followed by 2 weeks of methyl cellulose culture to obtain colony frequencies. This lengthy time period is required because progenitor cells read out at earlier time points whereas the colonies counted at 7 weeks are believed

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to be derived from stem cells. The second limiting dilution assay measures the frequency of cobblestone area forming cells (CAFC) on a layer of stromal cells after 5 weeks of culture. The two main drawbacks of these assays are lengthy time period required and the subjectivity involved in scoring based on cell morphology.

All publications cited herein are hereby incorporated herein by reference in their entirety.

Summary of the Invention

The present invention encompasses methods of detecting and quantitating hematopoietic stem cells in a population of hematopoietic cells. A population of hematopoietic cells is first obtained and treated to enrich for stem cells. Single cells in the enriched population are deposited onto a suitable growth substrate such as a well in a 96 well microtiter plate. The single cell deposits are cultured under conditions suitable to maintain viability and induce differentiation. The resultant populations are then assayed for the expression of lineage-specific messenger RNA transcripts (hereinafter "transcripts") specific to at least two different lineages. The presence of transcripts specific to two different lineages is scored as positive for the initial presence of a stem cell.

Brief Description of the Drawing

Figure 1 is a plot depicting the capillary electrophoresis results of a reverse transcription coupled polymerase chain reaction sample for the CD19 transcripts.

Detailed Description of the Invention

The present invention relates to methods of detecting and quantitating stem cells in a population of hematopoietic cells enriched for stem cells. The cells are deposited as single cells on suitable stromal cells and cultured under

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conditions suitable to induce multilineage differentiation of stem cells. After a suitable time, the resulting cells are analyzed for the presence of mRNA transcripts of proteins from at least two different lineages. The assay is completed in 2-3 weeks and does not rely on subjective analysis of cobblestone area cell morphology to determine the stem cell activity. This analysis is extremely sensitive and has the capability for analysis of large numbers of samples in a relatively short time. The frequency of stem cells in the sample is calculated from the number of wells positive for transcripts from at least two different lineages. Since only primitive stem cells have pluripotential capability, results of this assay reflect the stem cell content of the original sample.

Typically, enrichment for stem cells relies on positive selection for cells bearing surface markers present on stem cells and/or negative selection to remove cells bearing surface markers absent on stem cells.

Stem cells have been characterized by the following phenotypes. In the case of fetal cells including, but not limited to: CD34⁺, CD34^{hi}, CD3⁻, CD7⁻, CD8⁻, CD10⁻, CD14⁻, CD15⁻, CD19⁻, CD20⁻, and Thy-1⁻. In the case of adult cells including, but not limited to: CD34⁺, CD34^{hi}, CD3⁻, CD7⁻, CD8⁻, CD15⁻, CD19⁻, CD20⁻, and Thy-1⁻ or as represented in Table 1. Also, rho123 can divide the cells into high and low subsets ("rho^{lo}" and "rho^{hi}"). See Spangrude (1989) *Immunol. Today* 10:344-350, for a description of the use of rho123 with mouse stem cells. Preferably the cells are rho^{lo}. Preferably, the stem cells are human but may derive from any suitable animal. LIN⁻ cells generally refer to cells which lack markers associated with T cells (such as CD2, 3, 4 and 8), B cells (such as CD10, 19 and 20), myeloid cells (such as CD14, 15, 16 and 33), natural killer ("NK") cells (such as CD2, 16 and 56), RBC (such as glycophorin A), megakaryocytes, mast cells, eosinophils or basophils. The absence or low expression of

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such lineage specific markers is identified by the lack of binding of antibodies specific to the cell specific markers, useful in so-called "negative selection". Analyses for hematopoietic progenitors have been reported by Whitlock and Witte (1982) *Proc. Natl. Acad. Sci. USA* 79:3608-3612; and Whitlock et al. (1987) *Cell* 48:1009-1021. As used herein, the preferred LIN⁻ panel is CD14 and CD15.

Table 1 summarizes probable phenotypes of stem cells in fetal, adult, and mobilized peripheral blood. In Table 1 myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells and AMPB stands for adult mobilized peripheral blood. As used herein both infra, supra and in Table 1, the negative sign or, uppercase negative sign, (-) means that the level of the specified marker is undetectable above Ig isotype controls by FACS analysis, and includes cells with very low expression of the specified marker.

Table 1															
Probable Stem Cell Phenotypes															
	NK and T cell markers				B cell markers				Myelomonocytic				Other		
	CD2	CD3	CD8		CD10	CD19	CD20	CD14	CD15	CD16	CD33	CD34	CD38	HLA-DR	Rho
FBM	+	+	+		+	+	+	+	+	+	?	+	+	+	+
ABM	+	+	+		+	+	+	+	+	+	+	+	+	+	+
AMPB	+	+	+		+	+	+	+	+	+	+/?	+	?	+/?	+

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Sources of cells for subsequent purification include, but are not limited to, bone marrow, both adult and fetal, mobilized peripheral blood (MPB), blood, umbilical cord blood, embryonic yolk sac, fetal liver, and spleen, both adult and fetal. Bone marrow cells may be obtained from any known source, including but not limited to, ilium (e.g. from the hip bone via the iliac crest), sternum, tibiae, femora, spine, or other bone cavities.

For isolation of bone marrow from fetal bone or other bone source, an appropriate solution may be used to flush the bone, including but not limited to, salt solution, conveniently supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient buffers include, but are not limited to, Hepes, phosphate buffers and lactate buffers. Otherwise, bone marrow may be aspirated from the bone in accordance with conventional techniques.

Various treatments may be employed to separate the cells. Preferably, cells of dedicated lineage are removed initially. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies may be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain "relatively crude" separations. Such separations are where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker may remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

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Procedures for separation may include but are not limited to, magnetic separation using antibody-coated magnetic beads; affinity chromatography; cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including but not limited to, complement and cytotoxins; and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique.

The use of separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye, Hoechst 33342).

Techniques providing accurate separation include but are not limited to, FACS, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

The antibodies can be conjugated to identifiable agents including, but not limited to, enzymes, magnetic beads, colloidal magnetic beads, haptens, fluorochromes, metal compounds, radioactive compounds or drugs. The enzymes that can be conjugated to the antibodies include, but are not limited to, alkaline phosphatase, peroxidase, urease and β -galactosidase. The fluorochromes that can be conjugated to the antibodies include, but are not limited to, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, phycoerythrin, allophycocyanins and Texas Red. For additional fluorochromes that can be conjugated to antibodies, see Haugland, *Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals* (1992-1994). The metal compounds that can be conjugated to the antibodies include, but are not limited to, ferritin, colloidal gold, and particularly, colloidal superparamagnetic beads. The

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haptens that can be conjugated to the antibodies include, but are not limited to, biotin, digoxigenin, oxazalone, and nitrophenol. The radioactive compounds that can be conjugated or incorporated into the antibodies are known to the art, and include but are not limited to technetium 99m (^{99m}Tc), ¹²⁵I and amino acids comprising any radionuclides, including, but not limited to, ¹⁴C, ³H and ³⁵S. Any compound known in the art that can be conjugated to the antibodies by any method known in the art is suitable for use in the present invention as long as the cells selected by such method retain viability and the ability to differentiate in vitro.

Other techniques for positive selection may be employed, which permit accurate separation, such as affinity columns, and the like. The method should permit the removal to a residual amount of less than about 20%, preferably less than about 5%, of the non-stem cell populations.

Cells may be selected based on light-scatter properties as well as their expression of various cell surface antigens. The purified stem cells have low side scatter and low to medium forward scatter profiles by FACS analysis. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a coarse separation, followed by a fine separation, with positive selection of a marker associated with the stem cells and negative selection for markers associated with lineage committed cells.

It has now been found that a reverse transcription coupled polymerase chain reaction (RT/PCR) assay detects transcripts for myeloid and lymphoid lineage-specific markers in progenitor cells derived from single stem cells. Once the populations enriched for stem cells have been isolated, they

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are deposited as single cells and propagated in vitro under conditions that promote multilineage differentiation.

Typically, stromal cells including, but not limited to, mouse, porcine or human cells are provided. Mouse stromal cells may come from various strains, including, but not limited to, AC3 or AC6 (otherwise referred to herein as "SyS1" or "AC6.21"). Stromal cells are derived from mouse, porcine or human bone marrow by selection for the ability to maintain human stem cells, and the like. Stromal cells can be obtained from bone marrow, fetal thymus or fetal liver, and provide for the secretion of growth factors associated with stem and progenitor cell maintenance and differentiation. Before using in the co-culture, the mixed stromal cell preparations may be freed of hematopoietic cells employing appropriate monoclonal antibodies for removal of the undesired cells, e.g., with antibody-toxin conjugates, antibody and complement, etc. Alternatively, cloned stromal cell lines may be used where the stromal lines may be allogeneic or xenogeneic.

The population enriched for stem cells is preferably deposited into individual wells of a 96 well plate using an automated cell deposition unit (ACDU) on a fluorescence activated cell sorter (FACS), although any method known in the art may be used. Preferably, the cells are CD34⁺Thy-1⁺LIN⁻. These single cells are then preferably co-cultured on a mouse stromal line (AC6.21) with cytokines in amounts sufficient to induce differentiation of stem cells. Preferable cytokines and concentrations are provided in the examples; however, any cytokine and concentration thereof which maintains viability and induces differentiation is suitable. For instance, a suitable medium is basal media, e.g., RPMI or IMDM or a 1:1 combination supplemented with fetal calf serum (FCS). Cells can also be grown in myeloid long term culture medium (Stem Cell Technologies, Inc.) consisting 12.5% FCS, 12.5% horse serum, 1×10^{-4} M 2-

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mercaptoethanol in alpha medium supplemented with 2 mM L-glutamine, 0.2 mM inositol and 20 mM folic acid. Another suitable growth medium is 10% FCS, 1:1 RPMI/IMDM, 2 mM L-glutamine, 1×10^{-4} M 2-mercaptoethanol, and 1 mM sodium pyruvate.

During the first part of the culture period, cytokines known to act on the most primitive cells will be included in the culture medium. Preferably, the cytokines include, but are not limited to, at least one of interleukin 3 (IL3), interleukin 6 (IL6), granulocyte macrophage colony stimulating factor (GMCSF), granulocyte colony stimulating factor (GCSF), stem cell factor (SCF), insulin-like growth factor 1 (IGF1), basic fibroblast growth factor (bFGF), interleukin 7 (IL7), interleukin 4 (IL4), and interleukin 11 (IL11). More preferably, the cytokines include IL3, IL6, GMCSF and SCF. Most preferably, the cytokines include IL3, IL6, GMCSF, SCF, IGF and bFGF during the first week of culture. The second and third week of culture is performed in the presence of cytokines acting on cells at later stages of differentiation and will drive differentiation into more than one specific lineage. Suitable cytokines are those enumerated above with the exception of IL3. Preferably, the cytokines are IL6, IL7, GMCSF and SCF. More preferably, the cytokines are IL6, IL7, GMCSF, SCF, IGF1 and bFGF.

The cells are then harvested, the RNA extracted and amplified for transcripts which encode specific markers for at least two lineages. Preferably, the lineage-specific transcripts are those occurring early in differentiation. The lineage-specific transcripts may encode erythroid, lymphoid or myeloid specific proteins. Preferably, the lymphoid-specific transcripts include, but are not limited to, those encoding CD19, B220, RAG1, CD20 or RAG2. Preferably, the lymphoid transcript is that encoding CD19. Preferably, the myeloid transcripts include, but are not limited to, that encoding CD33. Preferably, the erythroid-

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specific transcripts include, but are not limited to, those encoding spectrin, GATA1 and β -globin.

Conversion of mRNA into cDNA is accomplished by any suitable reverse transcriptase enzyme, preferably, Moloney-murine leukemia virus (M-MLV) derived enzyme. The oligonucleotide primers necessary for this reaction include, but are not limited to, oligo dT primers, random primers or specific downstream primers. Preferably, the primer is oligo dT.

Any suitable sets of oligonucleotide primers may be used. Preferably, the oligonucleotide primers specific to CD19 have the following sequences: 5'-ATGTGGGTAATGGAGACGGGTC-3'; and 5'-TGGGGTCAGTCATTTCG CTTTC-3'. Preferably, the oligonucleotide primers specific to CD33 have the sequences: 5'-TTACAGCCCT GCTCGCTCTTTG-3'; and 5'-CAAGAATCAGCCTTTGGTCCC -3'. A second, subsequent, step of analysis may be performed using a set of nested primers to CD19 cDNA sequences. Preferably, the nested oligonucleotide primers for the CD19 cDNA have the sequences: 5'-ATTGTCACCG TGGCAACCTGAC-3'; and 5'-GGACCAGGGCTCTTTGAAGATG-3'. The nested PCR amplification is not limited to CD19 and may include any lineage specific marker.

Preferably, the amplification of transcripts is by reverse transcription coupled polymerase chain reaction, although any method of RNA amplification or RNA coupled DNA amplification known in the art may be used. These include, but are not limited to, the self-sustained sequence replication (3SR) and branched DNA (bDNA) amplification methods.

Analyses of the amplified transcripts may be performed by any method known in the art, including, but not limited to, capillary electrophoresis, agarose gel electrophoresis, fluorescence of fluorescently tagged primers, binding of biotinylated primers and high performance liquid

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chromatography. Preferably, the monitoring is by capillary electrophoresis.

The examples presented below show that sorted CD34⁺Thy-1⁺LIN⁻ stem cells from bone marrow have been examined in both the single cell two week transcript analysis assay and a typical CAFC assay. At two weeks, 44/190 wells had evidence of colony formation, and 16 of these were analyzed for transcripts for CD33 and for CD19. CD19 transcripts were detected in 16/16 colonies, and CD33 transcripts were detected in 10/16 colonies. The frequency of single cell-derived colonies with expression of both CD33 and CD19 was $10/16 \times 44/190 = 14\% = 1/7$. The frequency of CAFC obtained at 5 weeks from the same sorted cell cultures in IL6 and LIF on AC6.21 stromal cells was 1/12.7. Correlation of stem cell assay readout at two weeks with the established 5 week CAFC yields a practical QC analysis of potency for clinical stem cell transplantation.

The following examples are provided to illustrate but not limit the invention.

Example 1

Cell Selection and Culture

Bone Marrows harvested from normal adults were subjected to Ficoll-Hypaque density gradient separation (1.077 g/cm³) and bone marrow mononuclear cells were selected for CD34⁺ cells by incubating with QBEND10 anti-CD34 antibody (IOM34, AMAC, Inc.) followed by Dynabeads M450 sheep anti-mouse IgG1 (DynaI Inc., Oslo, Norway). At the end of the incubations, CD34⁺ cells were bound to the Dynabeads through the interaction between anti-CD34 antibody and sheep anti-mouse IgG1. The unbound cells were removed by pipetting while retaining the cells bound to Dynabeads by placing the tube containing the cells against a magnet. Finally CD34⁺ cells were released from the Dynabeads by enzymatic digestion with O-sialoglycoprotein endopeptidase (Accurate Chemical,

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Westbury, New York). The CD34⁺ cells were stained with monoclonal anti-CD34 PR3-sulforhodamine, anti CD14 PR4-fluorescein, anti-CD15 PR9-Fluorescein, anti-CDw90 (Thy-1) PR13-biotin, streptavidin and biotin phycoerythrin. Fluorescently stained cells were sorted for CD34⁺Thy-1⁺LIN⁻ (LIN⁻ is CD14⁻ and CD15⁻) stem cells and individual cells were deposited into wells of a 96-well plate at a concentration of one cell per well using an automated cell deposition unit on the FACS.

These single cells were co-cultured on a mouse stromal cell line (AC6.21, a clonal passage of AC6) at 37°C in humidified air plus 5% CO₂ according to the method described by Baum et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2804-2808. The culture medium contained 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 5 x 10⁻⁵ M β-mercaptoethanol (β-ME), IMDM/RPMI (50%/50%), 1% Penicillin/Streptomycin, interleukin-3 (IL3, Sandoz, 10 ng/ml), IL-6 (Sandoz, 10 ng/ml), insulin like growth factor-1 (IFG-1, R&D Systems, 10 ng/ml), basic fibroblast growth factor-1 (bFGF, R&D System, 10 ng/ml), stem cell factor (SCF, R&D Systems, 50 ng/ml) and granulocyte macrophage colony stimulating factor (GMCSF, 10 ng/ml). After one week of culture, the cytokine combination was changed to IL6 (10 ng/ml), IL7 (R&D Systems, 10 ng/ml), IFG-1 (10 ng/ml), bFGF-1 (10 ng/ml), SCF (50 ng/ml) and GMCSF (10 ng/ml). Cells were grown in this cytokine combination for 1-2 additional weeks before harvesting the cells for reverse transcription coupled polymerase chain reaction (RT/PCR). Cells were fed once a week by replacing 80% of the old media with fresh media containing the appropriate cytokines.

To determine the percentage of stem cells present initially, a 96 well culture plate was set up in a limiting dilution format ranging from 100 cells per well to 0.78 cells per well which was utilized to determine the stem cell frequency based on the cobblestone area forming cell (CAFC)

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morphology determination assay as described by Ploemacher et al. (1989) *Blood* 74:2755-2653. The cells in this plate were maintained with AC6.21 and the same culture media as the single cell plates except only IL-6 and leukemia inhibitory factors LIF (10 ng/ml each) were used as cytokines. This plate was scored for CAFCS after 5 weeks of culture. CAFC frequency was calculated from the fraction of nonresponding cultures, using Poisson distribution. The CAFC assay also allowed validation of the RT/PCR based assay for stem cell frequency in a given specimen.

Example 2

RNA isolation and RT/PCR

After 2-3 weeks of cell culture, RNA was isolated from each of the 96 wells showing cell growth. This was performed by aspirating culture media and adding 100 μ L of RNA STAT60™ solution (Tel-Test "B" Inc, Friendswood, TX) to extract RNA from small numbers of cells. Extraction and precipitation of RNA was performed according to the protocol provided by the manufacturer. RNA was also isolated from AC6.21, KG1A and Daudi cell lines to be utilized in the control reactions. RNA obtained from each well was reverse transcribed to convert mRNA into cDNA. The reverse transcription reaction was performed in a 20 μ L volume containing 1 μ M oligo d(T)₁₈, 1 mM dNTPs, 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol (DTT), 10 Units of RNA inhibitor and 100 Units of RNase H minus M-MLV reverse transcriptase (Promega Corp., Madison, WI). The reactions were performed at 42°C for 30 minutes followed by 90°C incubation for 5 minutes. PCR reactions were performed according to the method described by Saiki et al. (1988) *Science* 239:487-490. Briefly, 70 μ L of PCR master mix were dispensed directly to the 20 μ L RT reaction volume. The final 100 μ L PCR reaction contained 2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.5 Units of AmpliTaq DNA polymerase (Perkin Elmer Corp.),

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0.25 μ M each CD19 and CD33 primers. The primer sequences for CD19 and CD33 are as follows:

CD19F3 primer 5'-ATGTGGGTAATGGAGACGGGTC-3'

CD19B7 primer 5'-TGGGGTCAGTCATTGCTTTC -3'

CD33F5 primer 5'-TTACAGCCCTGCTCGCTCTTTG-3'

CD33B13 primer 5'-CAAGAATCAGCCTTTGGTCCC -3'

PCR reactions were performed in a Perkin Elmer thermocycler 9600 machine by incubating the samples at 95°C, 1 min. followed by 95°C, 10 sec.; 56°C, 20 sec.; 72°C, 20 sec. (35 cycles), and finally 72°C, for 5 minutes. A second round of PCR was performed on all samples using a CD19 nested primer set to further amplify CD19 transcripts that are present in low copy numbers. Nested PCR reactions were performed using 2 μ L of the first round reaction volume utilizing the following CD19 primer set:

CD19F4 primer 5'-ATTGTCACCGTGGCAACCTGAC-3'

CD19B5 primer 5'-GGACCAGGGCTCTTTGAAGATG-3'

Reaction conditions employed were similar to the first round PCR except 20 cycles were performed. Reaction products were analyzed by gel electrophoresis and capillary electrophoresis.

Example 3

Analysis of PCR Products by Capillary Electrophoresis

First and second round PCR products were desalted using Q1Aquick™ PCR purification columns (Qiagen, Inc. Chatsworth, CA) before performing capillary electrophoresis. Samples were separated on a Biofocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA) using a 24 cm x 50 cm coated capillary and 0.267 M Tris-Borate buffer, pH 8.3

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with polymer modifier (PCR buffer), 0.01% sodium azide. Separation conditions were 60 seconds column wash with PCR buffer followed by electrophoretic sample injection at 8 kV for 8 seconds (polarity negative to positive), 15 minutes run at 8 kV with inlet and outlet reagent being the PCR buffer. Products were detected by a UV detector at 260 nm wavelength. Figure 1 shows an example of a capillary electrophoresis read-out that is positive for the CD19 amplification product.

FACS sorted CD34⁺Thy⁻LIN⁻ stem cells from bone marrow have been examined in both the single cell two week transcript analysis assay and the CAFC assay. At two weeks, 44/190 wells had evidence of colony formation, and 16 of these were analyzed for CD33 and CD19. CD19 transcripts were detected in 16/16 colonies, and CD33 transcripts were detected in 10/16 colonies. The frequency of single cell-derived colonies with expression of both CD33 and CD19 was $10/16 \times 44/190 = 14\% = 1/7$. The frequency of CAFC obtained at 5 weeks from the same sorted cell cultures in IL-6 and LIF on AC6.21 stromal cells was 1/12.7.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention which is delineated by the appended claims.

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Claims

1. A method of detecting the presence of a hematopoietic stem cell in a population of hematopoietic cells comprising the steps of:

- a) obtaining a population of hematopoietic cells;
- b) treating the population of hematopoietic cells to obtain a population enriched for stem cells;
- c) depositing single cells from the population enriched for stem cells onto a suitable growth substrate;
- d) culturing the deposited cells under conditions suitable to maintain viability and induce differentiation; and

e) detecting the expression of lineage-specific transcripts specific to at least two different lineages in the deposited cells,

wherein the presence of transcripts to two different lineages in the differentiated cells is scored as positive for the initial presence of a stem cell.

2. The method according to claim 1 wherein the sorting step is by fluorescence activated cell sorting (FACS).

3. The method according to claim 1 or 2 wherein the hematopoietic cells are sorted on the basis of cell surface markers.

4. The method according to claim 3 wherein the cell surface markers are selected from the group consisting of CD34⁺, Thy-1⁺ and lack of at least one lineage-specific marker (LIN⁻).

5. The method according to claim 4 wherein the LIN⁻ markers are selected from the group consisting of CD2, CD14, CD15, CD16, CD19 and glycophorin A.

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6. The method according to claim 4 wherein the sorted cells are CD34⁺Thy-1⁺LIN⁻.

7. The method according to any one of claims 1-6 wherein the depositing step deposits single cells into a well of a ninety-six well microtiter plate.

8. The method according to claim 7 wherein the depositing is by an automated cell deposition unit (ACDU) on a FACS.

9. The method according to any one of claims 1-8 wherein the cell population enriched for stem cells is cultured in the presence of stromal cells.

10. The method according to any one of claims 1-9 wherein the cell population enriched for stem cells is cultured in the presence of cytokines in amounts sufficient to induce differentiation of stem cells.

11. The method according to claim 10 wherein the cytokines are selected from the group consisting of interleukin 3 (IL3), interleukin 6 (IL6), granulocyte macrophage colony stimulating factor (GMCSF), stem cell factor (SCF), insulin-like growth factor 1 (IGF1), basic fibroblast growth factor (bFGF), interleukin 7 (IL7), interleukin 4 (IL4), and interleukin 11 (IL11).

12. The method according to claim 10 or 11 wherein the first culturing step is in the presence of IL3, IL6, GMCSF and SCF.

13. The method according to claim 12 further comprising culturing in the presence of IGF1 and bFGF.

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14. The method according to any one of the preceding claims wherein the culturing step further comprises a second, subsequent, culturing step under conditions suitable to maintain viability and induce differentiation of stem cells.

15. The method according to claim 14 wherein the second culturing step is in the presence of cytokines in amounts sufficient to induce differentiation of stem cells.

16. The method according to claim 15 wherein the cytokines are selected from the group consisting of interleukin 6 (IL6), granulocyte macrophage colony stimulating factor (GMCSF), stem cell factor (SCF), insulin-like growth factor 1 (IGF1), basic fibroblast growth factor (bFGF), interleukin 7 (IL7), interleukin 4 (IL4), and interleukin 11 (IL11).

17. The method according to claim 16 wherein the cytokines are selected from the group consisting of IL6, IL7, GMCSF and SCF.

18. The method according to claim 17 further comprising culturing in the presence of IGF1 and bFGF.

19. The method according to any one of claims 14-18 wherein the first culturing step is for about one week and the second culturing step is for about two weeks.

20. The method according to any one of the preceding claims wherein the lineage-specific transcripts are selected from those appearing early in differentiation.

21. The method according to claim 20 wherein the lineage-specific transcripts are selected from the group consisting of erythroid, lymphoid and myeloid.

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22. The method according to claim 21 wherein the transcripts are selected from the group consisting of CD19, B220, RAG1 and RAG2.

23. The method according to claim 22 wherein the transcript is CD19.

24. The method according to claim 23 wherein the CD19 transcript is detected using oligonucleotides having the sequence:

5'-ATGTGGGTAATGGAGACGGGTC-3'; and
5'-TGGGGTCAGTCATTTCGCTTTC -3'.

25. The method according to claim 21 wherein the transcript is CD33.

26. The method according to claim 25 wherein the CD33 transcript is detected using oligonucleotides having the sequence:

5'-TTACAGCCCTGCTCGCTCTTTG-3'; and
5'-CAAGAATCAGCCTTTGGTCCC -3'.

27. The method according to claim 21 wherein the lineage-specific marker is erythroid and is selected from the group consisting of spectrin, GATA 1 and β -globin.

28. The method according to claim 26 further comprising a second, subsequent, step of RT/PCR using a set of nested primers to a myeloid or lymphoid transcript.

29. The method according to claim 28 using oligonucleotides having the sequence:

5'-ATTGTCACCGTGGCAACCTGAC-3'; and
5'-GGACCAGGGCTCTTTGAAGATG-3'.

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30. The method according to any of the preceding claims wherein the detection step is by capillary electrophoresis.

31. An oligonucleotide selected from:

5'-ATGTGGGTAATGGAGACGGGTC-3',
5'-TGGGGTCAGTCATTCGCTTTC -3',
5'-TTACAGCCCTGCTCGCTCTTTG-3',
5'-CAAGAATCAGCCTTTGGTCCC -3',
5'-ATTGTCACCGTGGCAACCTGAC-3', and
5'-GGACCAGGGCTCTTTGAAGATG-3'.

32. The use of an oligonucleotide according to claim 31 in a method for detecting stem cells, e.g., according to any one of claims 1-30.

1/1

Figure 1

